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Transcription factor DREF regulates expression of the microRNA gene *bantam* in *Drosophila melanogaster*

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Abstract. The *bantam* gene encodes a vital microRNA and has a complex expression pattern in various tissues at different stages of Drosophila development. This microRNA is involved in the control of normal development of the ocular and wing imaginal discs, the central nervous system, and also in maintaining the undifferentiated state of stem cells in the ovaries of adult females. At the cellular level, *bantam* stimulates cell proliferation and prevents apoptosis. The *bantam* gene is a target of several conserved signaling cascades, in particular, Hippo. At the moment, at least ten proteins are known to directly regulate the expression of this gene in different tissues of Drosophila. In this study, we found that the *bantam* regulatory region contains motifs characteristic of binding sites for DREF, a transcription factor that regulates the expression of Hippo cascade genes. Using transgenic lines containing a full-length *bantam* lethality-rescuing deletion fragment and a fragment with a disrupted DREF binding site, we show that these motifs are functionally significant because their disruption at the *bantam* locus reduces expression levels in the larvae and ovaries of homozygous flies, which correlates with reduced vitality and fertility. The effect of DREF binding to the promoter region of the *bantam* gene on its expression level suggests an additional level of complexity in the regulation of expression of this microRNA. A decrease in the number of eggs laid and a shortening of the reproductive period in females when the DREF binding site in the regulatory region of the *bantam* gene is disrupted suggests that, through *bantam*, DREF is also involved in the regulation of Drosophila oogenesis.
Key words: microRNA; genetic regulation; mutagenesis; transcription; transcription factors.

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Транскрипционный фактор DREF регулирует экспрессию гена микроРНК *bantam Drosophila melanogaster*

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Аннотация. Ген *bantam* кодирует жизненно важную микроРНК и имеет сложный паттерн экспрессии в различных тканях на разных стадиях развития дрозофилы. Эта микроРНК обеспечивает нормальное развитие глазных и крыловых имагинальных дисков, центральной нервной системы, а также участвует в поддержании недифференцированного состояния стволовых клеток в яичниках взрослых самок. На клеточном уровне *bantam* стимулирует пролиферацию клеток и препятствует апоптозу. Ген *bantam* является мишенью нескольких консервативных сигнальных каскадов, в частности Hippo. На сегодняшний день известно не менее 10 белков, напрямую регулирующих экспрессию этого гена в разных тканях дрозофилы. В настоящей работе мы обнаружили, что регуляторная область *bantam* содержит мотивы, характерные для сайтов связывания DREF – транскрипционного фактора, который регулирует экспрессию генов каскада Hippo. Используя трансгенные линии, содержащие полноразмерный фрагмент, спасающий летальность делеции *bantam*, и фрагмент с нарушенным сайтом связывания DREF, мы показали, что эти мотивы имеют функциональное значение, поскольку их нарушение в локусе *bantam* снижает уровень экспрессии в личинках и яичниках гомозиготных мух, что коррелирует со сниженной жизнеспособностью и фертильностью. Влияние связывания DREF с промоторной областью гена *bantam* на уровень его экспрессии предполагает дополнительный уровень сложности регуляции экспрессии этой микроРНК. Снижение количества откладываемых яиц и сокращение репродуктивного периода у самок при нарушении сайта связывания DREF в регуляторной области гена *bantam* позволяют предполагать, что через *bantam* DREF также участвует в регуляции оогенеза дрозофилы.
Ключевые слова: микроРНК; генетическая регуляция; мутагенез; транскрипция; факторы транскрипции.

Introduction

The *bantam* gene encodes a vital microRNA that is expressed in many tissues throughout the Drosophila life cycle. At the cellular level, *bantam* stimulates cell proliferation and prevents apoptosis (Brennecke et al., 2003). This microRNA provides normal development of the ocular and wing imaginal discs, the central nervous system, and it is also involved in maintaining the undifferentiated state of stem cells in the ovaries of adult females (Shcherbata et al., 2007; Peng et al., 2009; Reddy, Irvine, 2011; Slattery et al., 2013; Weng, Cohen, 2015).

Expression of the *bantam* gene is controlled by a wide range of transcription factors that form different assemblies in different tissues at different stages of development. Such conserved morphogens as *Notch*, *Wingless*, and *Dpp* are involved in the regulation of the *bantam* gene expression in different tissues (Herranz et al., 2008; Oh et al., 2010; Ku, Sun, 2017). Currently, about ten proteins that directly regulate *bantam* expression in different tissues are known. Dysregulation of *bantam* leads to abnormal development of many imaginal organs, and problems with vitality and fertility (Hipfner et al., 2002; Brennecke et al., 2003; Shcherbata et al., 2007).

One of the key regulators of *bantam* expression is the transcriptional coactivator Yorkie (Yki), which provides the necessary level of *bantam* expression by binding to tissue-specific transcription factors (Peng et al., 2009; Slattery et al., 2013; Nagata et al., 2022). Yki is part of the highly conserved Hippo signaling cascade (Oh et al., 2010). In Drosophila, this cascade suppresses the process of cell division, inducing cell cycle arrest and apoptosis. The cascade begins with the kinase Hippo (Hpo), which triggers the sequential phosphorylation of a number of proteins and, finally, leads to the inactivation of Yki (Huang et al., 2005; Reddy, Irvine, 2011). The *hpo* gene promoter has been shown to contain DRE motifs (DREF responding element). The promoter of the *warts* (*wts*) gene, the second key kinase of the Hippo cascade, also contains DRE motifs. The transcription factor DREF binds to these motifs, enhancing the expression of *hpo* and *wts* in the ocular imaginal discs (Fujiwara et al., 2012; Vo et al., 2014). Thus, DREF positively regulates the Hippo cascade and, accordingly, reduces the expression level of *bantam*.

In our work, we found that the *bantam* regulatory region contains motifs characteristic of DREF binding sites and tested the consequences of their disruption at the organism level. We examined how disruption of DREF binding sites at the *bantam* locus affected fly viability and fertility.

Materials and methods

Obtaining a mutation of the DREF binding site. To obtain the mutation, we used a DNA fragment from the *bantam* locus of 4709 bp in length (3L:637635-642343, release=r6.23), including DRE and DRE-like motifs. The DRE and DRE-like motifs were isolated from each other using the unique EcoRV restriction site located between them. To mutate each of these motifs, restriction with endonuclease ClaI (AT|CG|AT) was performed, and then the 5′-overhangs were end-filled using a Klenow fragment. This involved the insertion of two nucleotides (CG) into the key part of each motif. Then both parts of the 4709 bp DNA fragment with mutated DRE and DRE-like motifs were ligated and the resulting DNA fragment DREF was inserted at the KpnI and NotI restriction sites into the previously obtained pUni-mod vector containing the attB recombination site (Andreenkov et al., 2016). To obtain the transgenic line "*DREF*" we used the attP/attB system for specific integration, and a line containing an attP site in the region 10A1-2 of the X chromosome (Andreenkov et al., 2016).

Fly stocks. The "*4.7*" transgenic line's genotype was y^1, Df(1)w^{67c23}, 10A1-2-"4.7"; $ban^{\Delta 1}$/*TM6B*. It contained a transgene insertion with a full-length DNA fragment from the *bantam* locus with a length of 4709 bp into the 10A1-2 region (Schwartz et al., 2019).

The transgenic line "*DREF*" had the following genotype: y^1, Df(1)w^{67c23}, 10A1-2-"DREs_mut"; $ban^{\Delta 1}$/*TM6B*, and it contained the insertion of a transgene with a modified fragment 4.7 in the 10A1-2 region. The modification of the 4.7 fragment consisted in mutations in potential binding sites for the DREF protein. In all experiments performed, homozygotes for transgene insertion were studied.

The following lines were used as control: "*yw*" with the genotype y^1 Df(1)w^{67c23}; "*ban*+" with the genotype y^1, Df(1) w^{67c23}; +/*TM6B*, where *TM6B* is a balancer chromosome with *Tb* phenotype – short body of larvae and adults; "*Δban*" with the genotype y^1, Df(1)w^{67c23} ; $ban^{\Delta 1}$/*TM6B*.

Lines of flies were kept at +23 °C on standard food with addition of dry yeast.

Immunostaining. Indirect immunofluorescence staining of polytene chromosomes was performed according to the previously described protocol (Kolesnikova et al., 2013) using mouse monoclonal antibodies to the DREF protein (kindly provided by C.M. Hart, USA), diluted in a ratio of 1:200, with subsequent coloring by goat-anti-mouse-Alexa 488 antibodies (Thermo Fisher Scientific, # A28175), diluted in a ratio of 1:600.

Fly viability assessment. To determine the viability of transgenic line flies, 5 females and 5 males of the same line were placed in one glass vial. Once every 5 days, the flies were transferred to fresh food, and the experiment continued for a month. Flies of the "*TM6*" line were used as a control. Based on the results of 3 repetitions during the experiment, each line had on average the following number of descendants: "*4.7*" – 454 ± 92; "*DREF*"– 287 ± 112; "*TM6*" – 756 ± 289.

The viability of homozygotes for the ban$^{\Delta 1}$ deletion in transgenic lines was determined as the ratio of the number of flies with a body of normal length (*Tb*+) to the total number of hatched offspring. Since the *Tb* phenotype is identified at the stages of larvae, pupae and adults, it is possible to distinguish flies lacking the balancer, homozygous for the ban$^{\Delta 1}$ deletion, from heterozygous flies at different stages of development. The "*ban*+" line, without a transgene and without a deletion, was used as a control. The viability of flies at different developmental stages in transgenic lines was compared with the viability of control flies using Student's *t* test, with a preliminary check for normal distribution using the Shapiro–Wilk test.

Female fertility assessment. To determine the fertility of females of transgenic lines, they were crossed with males of the "*yw*" line. Females of the "*yw*" line were used as control. In each cross we used 5 females and 5 males. Every day the flies were transferred to fresh food and the number of eggs laid was counted. The experiment continued until the death of the last transgenic female in a vial. The number of eggs during the entire experiment was normalized to the number

of females. The experiment was repeated three times. When plotting the fertility dynamics curve, the number of eggs laid by females each day was normalized to the current number of living females. Differences in fertility levels between lines were assessed using Student's *t* test, as well as the χ^2 test.

Determination of the expression level of mature microRNA *bantam*. The expression level of mature microRNA *bantam* was determined by quantitative PCR combined with a reverse transcription reaction (qRT-PCR), adapted for the study of microRNAs through the use of an extended stem-loop primer (Chen et al., 2005; Kramer, 2011). We used *U6* snRNA as a reference gene (Zhang et al., 2017). To obtain cDNA, we used 5 μg of total RNA, M-MuLV-PH revertase, and accompanying reagents according to the manufacturer's instructions (Biolabmix). Relative *bantam* gene expression was determined using the ΔΔCt method. qRT-PCR was carried out using a thermal cycler BioRad C-1000 (USA).

The experiment was done in two biological replicates. We used 30 μl of the following reaction mixtures: for *bantam* detection – 3 μl of 5 μM ban-F and ban-R primers, 3 μl of 2.5 μM TaqMan-ban probe, 3 μl of 10xAS buffer, 3 μl of 4 μM dNTP, 1 u. a. of Taq polymerase; for *U6* detection – 3 μl of 10 μM U6-F and U6-R primers, 3 μl of 2.5 μM TaqMan *U6* probe, 3 μl of 10xAS buffer, 3 μl of 4 μM dNTP, 1 u. a. of Taq polymerase, 3 μl of 10 mM $MgCl_2$ to the final concentration of Mg^{2+} equal to 2.5 mM for a reaction. The nucleotide sequences of the primers and probes used in the experiments are given in 5′→3′ orientation: ban-SL – gtcgtatccagtgcagggtccgaggtattcgcactg gatacgacaatcag, ban-F – cgccgggcatgagatcattttg, ban-R – cagt gcagggtccgaggt, TaqMan-ban – cgcactggatacgacaatcagcttt, U6-SL – gtcgtatccagtgcagggtccgaggtattcgcactggatacgacggc catgc, U6-F – gccgcatacagagaagatta, U6-R – agtgcagggtc cgaggta, TaqMan-U6 – ttcgcactggatacgacggccatgc.

Results and discussion

The *bantam* regulatory region contains DREF binding sites

To study the role of DREF in the regulation of *bantam* gene expression, we used transgenic fly lines. The "*4.7*" fly line contained a transgene insertion with a 4709 bp long DNA fragment from the *bantam* locus (Fig. 1, *a*) into the 10A1-2 region of the X chromosome. This fragment, hereafter referred to as fragment 4.7 (see Fig. 1, *b*), contains a sequence encoding the *bantam* miRNA hairpin as well as two putative promoters of the *bantam* gene (Brennecke et al., 2003; Qian et al., 2011). It has been shown previously that the 4.7 fragment rescues the lethal $ban^{\Delta 1}$ deletion, removing approximately 21 kb from the *bantam* locus (Schwartz et al., 2019). In the fragment 4.7, 1.2 kb upstream of the *bantam* hairpin, we found TATCGATA and TATCGATG motifs corresponding to DRE and DRE-like elements, respectively (Ohler et al., 2002). Both motifs are characteristic of binding sites for the transcription factor DREF (see Fig. 1, *c*).

The "*DREF*" fly line contained a transgene insertion with the 4.7 fragment with disrupted DREF binding sites. To disrupt the DREF binding sites, we introduced mutations in the DRE (DREF-responding element) and DRE-like motifs (see Fig. 1, *c*). It should be noted that DRE and DRE-like include CGATA motifs that form the binding site for the insulator protein BEAF-32. The mutations we introduced did not disrupt the CGATA motifs and, accordingly, did not destroy the BEAF-32 binding site. Immunolocalization on polytene chromosomes of Drosophila larvae showed that in line "*4.7*" in the distal part of the 10A1-2 band at the site of transposon insertion there was an additional DREF localization signal (Fig. 2, *b*, red arrow). At the same time, this additional signal was absent

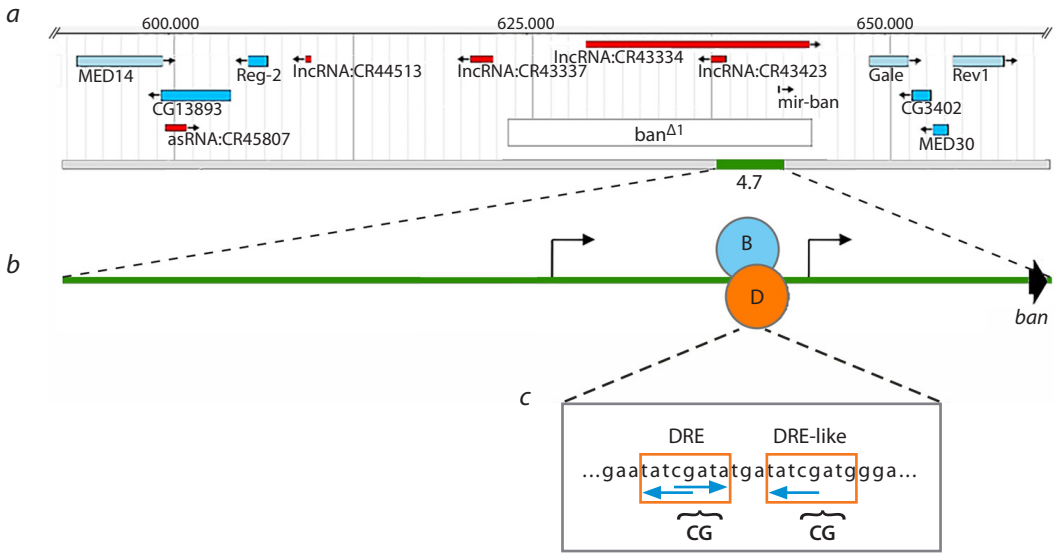

Fig. 1. Molecular and genetic organization of the *bantam* locus.

a – DNA fragment 4.7 containing the bantam gene (green rectangle), $ban^{\Delta 1}$ deletion (white rectangle). The location of other genes in the region (blue and red rectangles); *b* – the 4.7 DNA fragment organization scheme. Binding sites for DREF and BEAF-32 proteins (ovals D and B, respectively). Curved arrows correspond to the positions of putative promoters of the *bantam* gene (Brennecke et al., 2003; Qian et al., 2011). The *bantam* hairpin position (black arrow); *c* – nucleotide sequence with binding sites for DREF and BEAF-32 proteins. Motifs characteristic of the DREF (orange rectangles) and BEAF-32 (blue arrows) binding sites. Double nucleotide insertions that disrupt DREF binding sites are indicated by curly brackets.

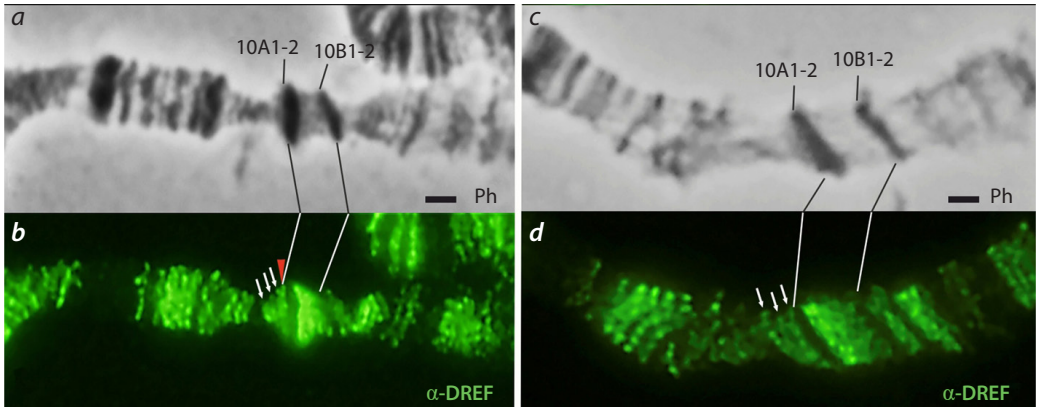

Fig. 2. Immunolocalization of the DREF protein in the 10A1-2 region of the X chromosome transgenic fly lines.

Fly lines: "*4.7*" (*a*, *b*) and "*DREF*" (*c*, *d*). Microphotographs of polytene X chromosomes of larval salivary glands in phase contrast (Ph) mode and after staining with antibodies against DREF (green). White arrows indicate endogenous DREF localization signals in the 9F region. The red triangle marks an additional localization signal of DREF in the distal part of the 10A1-2 band, corresponding to the localization of the transgene in the "*4.7*" line. There is no additional signal in the "*DREF*" line (*d*). Scale bar 1 μm.

in the "*DREF*" line (see Fig. 2, *d*). It can be assumed that the DRE and DRE-like motifs we found correspond to the binding site of the DREF protein, and the mutations of these motifs we introduced lead to disruption of DREF protein binding.

Disruption of the DREF binding site in the *bantam* regulatory region affects fly viability

We found that flies of the "*DREF*" line survived against the background of the ban$^{\Delta 1}$ deletion, but the viability of such flies was significantly reduced compared to flies of the "*4.7*" line (Fig. 3), as well as compared to control "*TM6*" flies, containing neither the transgene nor the ban$^{\Delta 1}$ deletion. Moreover, the death of flies homozygous for ban$^{\Delta 1}$ in the "*DREF*" line mainly occurred at the late pupal stage, which coincides with the characteristic lethality of the ban$^{\Delta 1}$ deletion (Brennecke et al., 2003). The use of control containing TM6B balancer allowed taking into account the influence of the balancer itself on the fly viability.

In order to determine whether the decrease in viability of transgenic "*DREF*" flies was associated with *bantam* gene expression, we examined the expression level of the mature *bantam* microRNA in the larvae of transgenic and control flies. The studies were carried out using Real-time PCR adapted for microRNA (Chen et al., 2005; Kramer, 2011). The "*yw*" line with a normal *bantam* locus served as a positive control. We used the "*Δban*" line with the ban$^{\Delta 1}$ deletion as negative control. Larvae homozygous for the insertions were selected. Since individuals homozygous for the ban$^{\Delta 1}$ deletion die at the pupal stage, studies were carried out on larvae. As expected, in the "*Δban*" line, the mature microRNA *bantam* was not detected in larvae homozygous for ban$^{\Delta 1}$ (Fig. 4). The expression of *bantam* was reduced in both transgenic lines, "*4.7*" and "*DREF*", compared to the control "*yw*". A significant decrease in the expression level of mature microRNA in the "*4.7*" line seems surprising given that the viability of flies in the "*4.7*" line did not differ from the control. This may be explained by the fact that although in the "*4.7*" line, the level of *bantam* microRNA expression was significantly reduced, it remained at a sufficient level in all the tissues where it was necessary for fly survival. And in the "*DREF*" line, the *bantam* expression level was ubiquitously at a low, threshold level, which significantly affected viability. It is also possible that in the "*DREF*" line, *bantam* expression was reduced only in certain tissues critical for fly survival. The data obtained suggest that although the "*DREF*" transgene rescues the ban$^{\Delta 1}$ deletion, it does not contain all the regulatory elements required for full *bantam* expression.

The fundamental ability of the mutant transgene in the "*DREF*" line to "rescue" the ban$^{\Delta 1}$ deletion indirectly confirms that the introduced mutations did not destroy the binding site of the BEAF-32 protein. We have previously shown that destruction of the BEAF-32 protein binding site in the *bantam* regulatory region leads to death at the late pupal stage (Schwartz et al., 2019).

Interestingly, in the "*DREF*" transgenic line, the viability of adult flies homozygous for ban$^{\Delta 1}$ was sex-dependent. The proportion of males homozygous for ban$^{\Delta 1}$ was only 30 % of

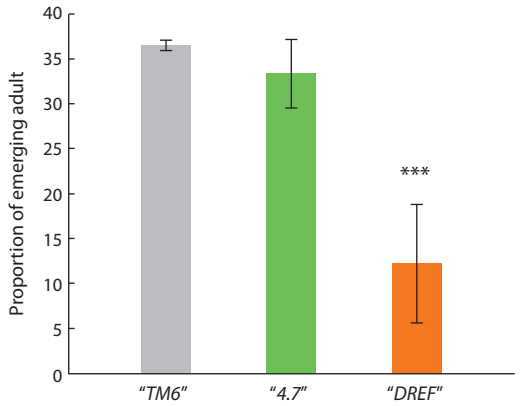

Fig. 3. Effect of DREF binding to the *bantam* regulatory region on adult fly viability in transgenic fly lines.

The proportion of adults homozygous for the ban$^{\Delta 1}$ deletion in transgenic fly lines "*4.7*" (green column) and "*DREF*" (orange column). The proportion of adults in the control fly line "*TM6*" (gray column), containing only native wild-type *bantam* locus. *** $p < 0.001$.

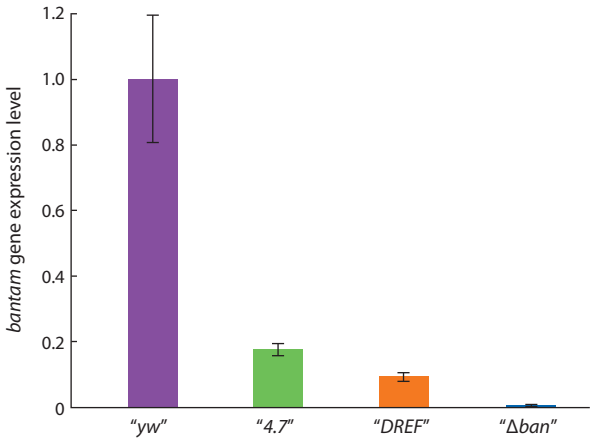

Fig. 4. The expression of *bantam* microRNA in the 3rd instar larvae.

The amount of mature microRNA *bantam* in the transgenic lines "*4.7*" and "*DREF*" and the control line "*Δban*", homozygous for the ban$^{\Delta 1}$ deletion. The expression of *bantam* in the control line "*yw*" was conditionally taken as one. The *bantam* expression levels were normalized to the expression level of the *U6* snRNA reference gene.

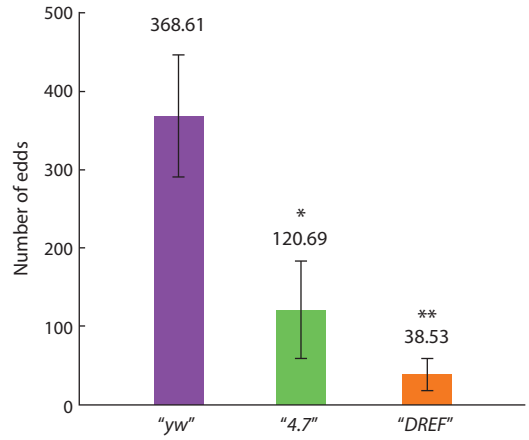

Fig. 5. Fertility of transgenic fly line females.

Average number of eggs per female from the control line "*yw*" and transgenic lines "*4.7*" and "*DREF*", homozygous for the transgenes and for the ban$^{\Delta 1}$ deletion. ** $p < 0.01$; * $p < 0.05$.

all adult flies, which is significantly lower than in the "*4.7*" line and in the control ($p<0.01$). This may be explained by the fact that disruption of the DREF binding site in the *bantam* regulatory region has a greater effect on the viability of males than females. Another explanation could be the different levels of transgene activity on the X chromosome associated with dosage compensation. Despite the fact that males of the "*DREF*" line, homozygous for ban$^{\Delta 1}$, were less viable than females, they did not have problems with fertility.

Disruption of the DREF binding site in the *bantam* promoter region significantly reduces female fertility

We studied the fertility of females of transgenic lines, estimating the average number of eggs laid per female (see Materials and methods). In the "*4.7*" line, the fertility of females homozygous for ban$^{\Delta 1}$ was significantly reduced compared to females of the "*yw*" control line and amounted to 32.7 % of the fertility of control "*yw*" females, taken as 100 % (Fig. 5). The fertility of females homozygous for ban$^{\Delta 1}$ in the "*DREF*" line was only 10.5 %. These data indicate that the 4.7 fragment does not contain all the regulatory elements necessary for the normal progression of oogenesis, and the mutation of the binding site in the "*DREF*" line disrupts this process even more crucially.

However, it should be taken into account that the life expectancy of females in transgenic lines was significantly reduced compared to control females of the "*yw*" line ($p<0.05$). Thus, in the "*yw*" line, 50 % of the females died on average on the 22nd day of the experiment, in the "*4.7*" line – on the 16th day, and in the "*DREF*" line – on the 10th day.

At the same time, a reduction in life expectancy was not the only explanation for the decrease in the number of eggs laid by females of transgenic lines. Analysis of the dynamics of egg laying showed that females of transgenic lines not only laid fewer eggs on each day of the experiment than females of the control "*yw*" line ($p<0.001$), but also finished laying eggs much earlier (Fig. 6). The reproductive period in females of the "*4.7*" line lasted on average 16.3 days ($p<0.01$), and in "*DREF*" females – 8.3 days ($p<0.001$), while the reproductive period in control "*yw*" females was 24.7 days.

Disruption of the DREF binding site reduces *bantam* miRNA expression in adult fly ovaries

The early completion of egg laying in transgenic lines is similar to the previously described situation with inactivation of *bantam* microRNA in germline stem cells in the ovaries of adult flies (Shcherbata et al., 2007). According to the authors, in such females, about 14 % of germline stem cells left their niche per day. This can lead to both a general decrease in fertility and a shortening of the reproductive period.

We decided to test whether DREF binding to the *bantam* regulatory region actually affects the expression level of mature microRNA. Using qRT-PCR, we showed that the expression of *bantam* microRNA in the ovaries of females of the transgenic lines "*4.7*" and "*DREF*" was lower than in females of the control "*yw*" line (Fig. 7).

The results obtained are consistent with the fact that in both transgenic lines the fertility of females was reduced compared to control "*yw*" females. Moreover, in the "*DREF*" line, *bantam* expression was reduced not only compared to "*yw*", but also compared to "*4.7*". This indicates that DREF binding to the regulatory region of the *bantam* gene plays an important role in its expression in the ovaries, and it is the disruption of this binding that may explain the significant decrease in fertility in females of the "*DREF*" line.

Conclusion

In this work, we tested the functionality of a potential binding site for the transcription factor DREF, found in the regulatory region of the *bantam* gene. Disruption of this DREF binding site has a significant impact on fly viability and female fertility. This is accompanied by a significant decrease in the expression of mature *bantam* microRNA in both whole larvae and the ovaries of adult flies. As was previously shown, DREF

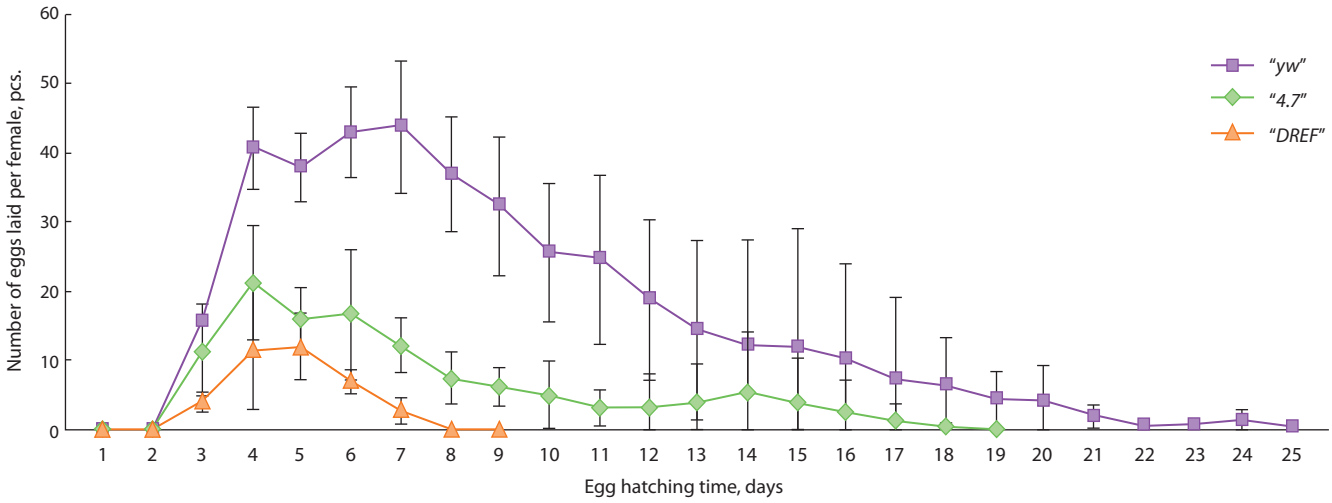

Fig. 6. Temporal dynamics of female fertility.

Average number of eggs laid per day per female in the control "*yw*" line and in the transgenic lines "*4.7*" and "*DREF*", homozygous for transgenes and for the ban$^{\Delta 1}$ deletion.

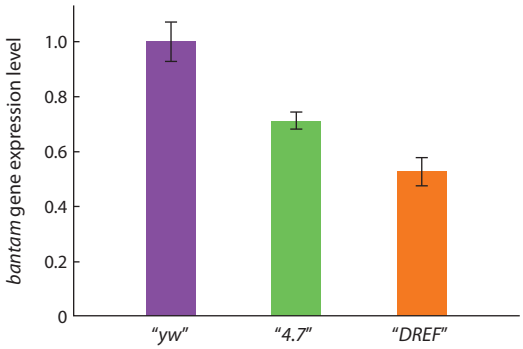

Fig. 7. The expression of mature *bantam* microRNA in the ovaries of transgenic females.

The amount of mature microRNA *bantam* in the transgenic lines "*4.7*" and "*DREF*", homozygous for the ban$^{\Delta 1}$ deletion. The expression of *bantam* in the control "*yw*" line was conditionally taken as one. The *bantam* expression levels were normalized to the expression level of the *U6* snRNA reference gene.

positively influences the activity of the Hippo cascade, thereby indirectly limiting the expression of *bantam* (Fujiwara et al., 2012; Vo et al., 2014). The effect of DREF binding to the promoter region of the *bantam* gene on its expression level suggests an additional level of complexity in the regulation of the expression of this microRNA.

A decrease in the number of eggs laid and a shortening of the reproductive period in females when the DREF binding site in the regulatory region of the *bantam* gene is disrupted suggests that DREF is also involved in the regulation of Drosophila oogenesis through *bantam*.

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